

PCT

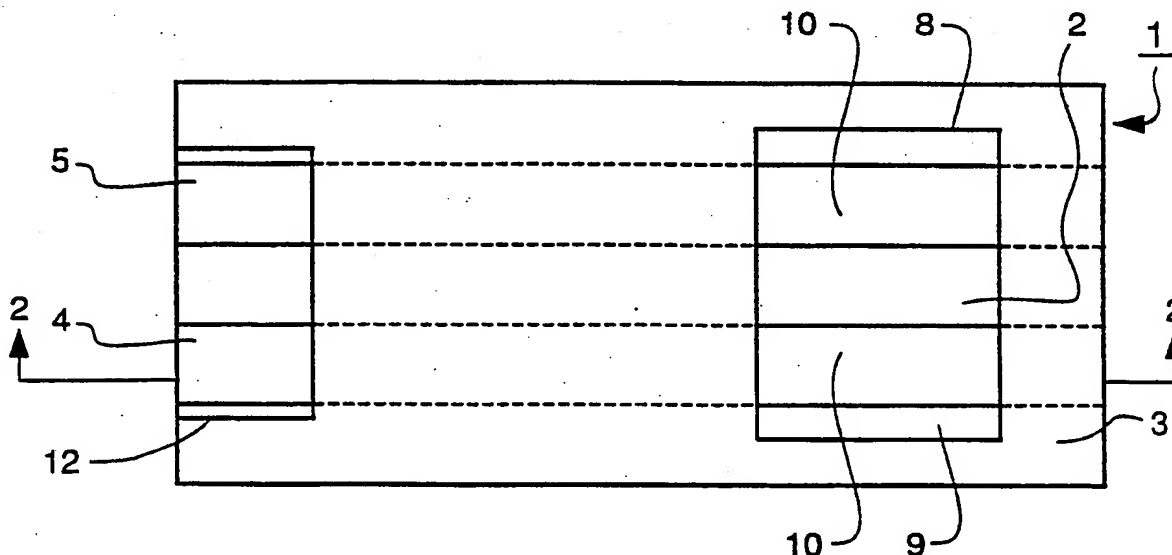
WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|   |           |   |
|---|-----------|---|
| <p>(51) International Patent Classification <sup>5</sup> :<br/>C12Q 1/54, C12M 1/36</p>   | <p>A1</p> | <p>(11) International Publication Number: WO 91/09139<br/>(43) International Publication Date: 27 June 1991 (27.06.91)</p>  |
| <p>(21) International Application Number: PCT/US90/07374<br/>(22) International Filing Date: 14 December 1990 (14.12.90)<br/>(30) Priority data: 451,671 15 December 1989 (15.12.89) US<br/>(71) Applicant: BOEHRINGER MANNHEIM CORPORATION [US/US]; P.O. Box 50528, Indianapolis, IN 46250-0528 (US).<br/>(72) Inventors: GERBER, Martin, T. ; 969 Nevele Lane, Carmel, IN 46032 (US). OCHS, M., Luann ; 8510 Chapel Pines Drive, Indianapolis, IN 46234 (US). KOST, Kent, M. ; 11229 Ashley Place, Fishers, IN 46038 (US). BATESON, Joseph, E. ; 6380 East 98th Street, Fishers, IN 46038 (US). WALLING, P., Douglas ; 10216 Seabreeze Way, Indianapolis, IN 46256 (US). POLLMANN, Klaus, H. ; Erfurter Weg 1, D-6823 Neulussheim (DE).</p> |           | <p>(74) Agent: YOUNG, D., Michael; Boehringer Mannheim Corporation, 9115 Hague Road, Indianapolis, IN 46250 (US).<br/>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).<br/><br/>Published<br/>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> |

(54) Title: REDOX MEDIATOR REAGENT AND BIOSENSOR



(57) Abstract

The invention provides an amperometric biosensor for detecting the concentration of an analyte in a fluid sample. The biosensor (1) has a working (4) and a counter electrode (5) fixed to an insulator material (7). A biosensor reagent (11) is placed in a well (9) so that it covers substantially all of the exposed surfaces (10) of the electrodes (4, 5). For testing, a sample containing the analyte is added to the reagent well (9), the reaction allowed to go to completion, a potential difference applied to the electrodes (4, 5) and the diffusion limited current is measured.

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                  | FI | Finland                               | ML | Mali                     |
| AU | Australia                | FR | France                                | MN | Mongolia                 |
| BB | Barbados                 | GA | Gabon                                 | MR | Mauritania               |
| BE | Belgium                  | GB | United Kingdom                        | MW | Malawi                   |
| BF | Burkina Faso             | GN | Guinea                                | NL | Netherlands              |
| BG | Bulgaria                 | GR | Greece                                | NO | Norway                   |
| BJ | Benin                    | HU | Hungary                               | PL | Poland                   |
| BR | Brazil                   | IT | Italy                                 | RO | Romania                  |
| CA | Canada                   | JP | Japan                                 | SD | Sudan                    |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SE | Sweden                   |
| CG | Congo                    | KR | Republic of Korea                     | SN | Senegal                  |
| CH | Switzerland              | LI | Liechtenstein                         | SU | Soviet Union             |
| CI | Côte d'Ivoire            | LK | Sri Lanka                             | TD | Chad                     |
| CM | Cameroon                 | LU | Luxembourg                            | TG | Togo                     |
| DE | Germany                  | MC | Monaco                                | US | United States of America |
| DK | Denmark                  | MG | Madagascar                            |    |                          |
| ES | Spain                    |    |                                       |    |                          |

## REDOX MEDIATOR REAGENT AND BIOSENSOR

Cross-References to Related Applications

This application is a continuation-in-part of U.S. Patent Application Number 07/451,671, filed December 15, 1989.

05 Field of the Invention

This invention relates generally to the determination of the concentration of analytes in fluids and more specifically to an amperometric biosensor for use in such determinations.

10 Background of the Invention

Biosensors are not new. Their use in the determination of concentrations of various analytes in fluids is also known.

15       Nankai et al., WO 86/07632, published December 31, 1986, discloses an amperometric biosensor system in which a fluid containing glucose is contacted with glucose oxidase and potassium ferricyanide. The glucose is oxidized and the ferricyanide is reduced to ferrocyanide. (This reaction is catalyzed by glucose oxidase.) After  
20       two minutes, an electrical potential is applied and a current caused by the re-oxidation of the ferrocyanide to ferricyanide is obtained. The current value, obtained a few seconds after the potential is applied correlates to the concentration of glucose in the fluid.

25       Because Nankai et al. discloses a method in which the reaction of glucose and ferricyanide may run to completion prior to the application of an electrical potential, this method is referred to as the "end-point" method of amperometric determination.

-2-

05     Nankai et al. discloses a system, wherein the glucose oxidase and potassium ferricyanide are held on a non-woven nylon mesh. The mesh is positioned so that it is in contact with a working electrode, a counter electrode and a reference electrode. The total surface area of the counter and reference electrodes is twice that of the working electrode.

10     Wogoman, EP 0 206 218, published December 30, 1986 discloses a biosensor having two electrodes, the electrodes being made of different electrically conducting materials. For example, the anode is formed from an anode material, such as platinum, and the cathode is formed from a cathode material, such as silver. The anode is coated with an enzyme. In a preferred embodiment, the coated  
15     electrode is covered with an elastomer that is permeable to glucose.

20     Pottgen et al., WO 89/08713, published September 21, 1989, discloses the use of a two electrode biosensor, wherein the electrodes are made of the same noble metal, but one of the electrodes (referred to as a pseudo-reference electrode) is larger than the other (working) electrode.

Conventional wisdom in the electrochemical arts suggests biosensors of the following types:

25         1) a three electrode system, wherein a working electrode is referenced against a reference electrode (such as silver/silver chloride) and a counter electrode provides a means for current flow;

30         2) a two electrode system, wherein the working and counter electrodes are made of different electrically conducting materials; and

-3-

3) a two electrode system, wherein the working and counter electrodes are made of the same electrically conducting materials, but the counter electrode is larger than the working electrode.

05       Conventional wisdom in the electrochemical arts does  
not suggest that a biosensor could include a two electrode  
system, wherein the working and counter electrodes are  
substantially the same size (or wherein the counter  
electrode is smaller than the working electrode) and made  
10 of the same electrically conducting material.

#### Summary of the Invention

The invention is a new biosensor (an electrochemical device) and method of its use. The biosensor includes working and counter electrodes of substantially the same  
15 size, made of the same electrically conducting material, and affixed to a first electrical insulator. Overlaying the electrodes is a second electrical insulator that includes a cutout portion that exposes substantially equal surface areas of the working and counter electrodes.

20       A reagent is added to the cutout portion. The reagent substantially covers the exposed electrode surfaces in the cutout portion and includes a redox mediator, an enzyme and a buffer.

25       When a sample containing an analyte is added to the reagent, the analyte, enzyme, and the redox mediator participate in a reaction, wherein the redox mediator is either reduced (receives at least one electron) or is oxidized (donates at least one electron). Usually, in  
30 this reaction, the analyte is oxidized and the redox mediator is reduced. After this reaction (wherein analyte is oxidized and redox mediator is reduced) is complete, an electrical potential difference is applied between the electrodes. The amount of oxidized form of the redox

-4-

mediator at the counter electrode and the applied potential difference must be sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode. After a short time delay, current produced by the electrooxidation of the reduced form of the redox mediator is measured and the observed current is correlated to the amount of the analyte in the sample.

Importantly, only two electrodes of substantially the same size and made of the same electrically conducting material are required when the reagent includes a sufficient amount of the oxidized form of the redox mediator to insure that the current produced during electrooxidation is limited by the oxidation of the reduced form of the redox mediator at the working electrode surface.

For current produced during electrooxidation to be limited by the oxidation of the reduced form of the redox mediator at the working electrode surface, the amount of oxidized form of the redox mediator at the surface of the counter electrode must always exceed the amount of reduced form of the redox mediator at the surface to the working electrode.

#### Brief Description of the Drawings

FIG. 1 is a schematic top view of a preferred embodiment of the inventive biosensor, excluding reagent and mesh covering.

FIG. 2 is a schematic elevation of the inventive biosensor of FIG. 1 along lines 2-2, and including reagent and mesh covering.

-5-

FIG. 3 is a schematic top view of a preferred embodiment of the inventive biosensor, including mesh covering.

Detailed Description of the Invention

05 Referring more specifically to FIGS. 1 through 3, there is shown the presently preferred embodiment of the biosensor of the present invention.

Biosensor 1 comprises first and second electrically insulating layers 2 and 3, respectively. Any useful  
10 insulating material will be suitable. Typically, plastics, such as vinyl polymers and polyimides provide the electrical and structural properties which are desired.

The biosensor shown in FIGS. 1 through 3 is  
15 intended to be mass produced from rolls of material, necessitating the selection of a material which is sufficiently flexible for roll processing and at the same time sufficiently stiff to give a useful stiffness to the finished biosensor.

20 Layers 2 and 3 may be of any useful thickness. In a preferred embodiment, layer 2 is about 360 microns thick and layer 3 is about 250 microns thick.

Working electrode 4 and counter electrode 5 are preferably deposited on a backing of insulator material 7,  
25 such as polyimide, to reduce the possibility of tearing the electrode before it is affixed to layer 2. Working electrode 4 and counter electrode 5 are substantially the same size and are made of the same electrically conducting material. Examples of electrically conducting materials  
30 that may be used are palladium, platinum, gold, silver, carbon, titanium, and copper. Noble metals are preferred because they provide a more constant, reproducible

-6-

electrode surface area. Palladium is particularly preferred because it is one of the more difficult noble metals to oxidize and because it is a relatively inexpensive noble metal. Silver is not preferred because it is more readily oxidized by air than the other noble metals listed above. Preferably, electrodes 4 and 5 are about 0.1 micron thick and backing 7 is about 25 microns thick (commercially available from Courtalls-Andus Performance Films in California and Southwall Technologies, Inc.) (Fig. 2).

Electrodes 4 and 5 must be sufficiently separated so that the electrochemical events at one electrode do not interfere with the electrochemical events at the other electrode. The preferred distance between electrodes 4 and 5 is about 1.2 millimeters (mm).

In the preferred embodiment, electrodes 4 and 5, affixed to backing 7, are unspooled from reels and attached to layer 2 by the use of hot melt adhesive (not shown). Electrodes 4 and 5 also preferably extend from one end of layer 2 to the other end in parallel configuration. (Fig. 1).

Insulating layer 3 is fixed on top of layer 2 and electrodes 4 and 5 by the use of hot melt adhesive (not shown). Layer 3 includes cut out portion 8, which defines reagent well 9 and exposes substantially equal surface areas 10 of electrodes 4 and 5.

In the preferred embodiment, cutout 8 is 4 mm by 6 mm and electrodes 4 and 5 are each 1.5 mm in width. Therefore, a surface area of about  $6 \text{ mm}^2$  is exposed for each of the two electrodes.

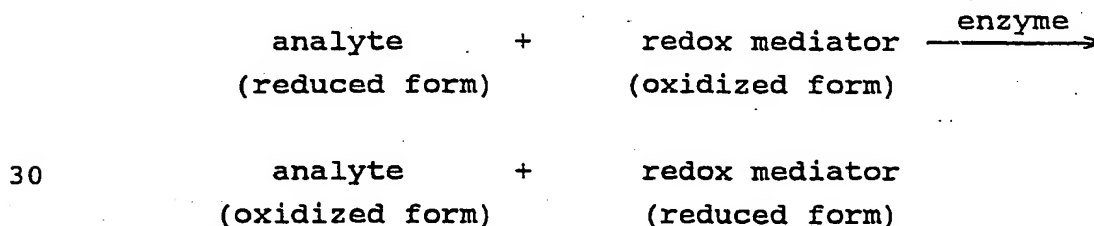
-7-

Biosensor 1 also includes a power source (not shown) in electrical connection with the working and counter electrodes and a current measuring meter (not shown) which is also in electrical connection with the working and counter electrodes.

Biosensor reagent 11 (Fig. 2) is placed in well 9 so that it covers substantially all of exposed surfaces 10 of electrodes 4 and 5 and preferably covers the exposed surface of layer 2 between the electrodes.

At a minimum, reagent 11 includes the oxidized form of a redox mediator, an enzyme, and a buffer. The oxidized form of the redox mediator must be of sufficient type to receive at least one electron from a reaction involving enzyme, analyte, and oxidized form of the redox mediator. (The term redox mediator means a mediator that is capable of undergoing an electrochemical, reversible oxidation-reduction reaction.) The enzyme must be of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte and oxidized form of the redox mediator. The buffer must be of sufficient type and in sufficient amount to provide and maintain a pH where the enzyme catalyzes the reaction involving enzyme, analyte and oxidized form of the redox mediator.

Generally, when a sample containing the analyte is added to the reagent, the analyte is oxidized and the oxidized form of the redox mediator is reduced as shown below:



-8-

The reaction shown above is permitted to go to completion. (Completion is defined as sufficient reaction involving analyte, enzyme, and redox mediator (oxidized form) to correlate analyte concentration to diffusion limited current generated by oxidation of the reduced form of the redox mediator at the surface of the working electrode.) After reaction is complete, a power source (e.g., a battery) applies a potential difference between the electrodes. When the potential difference is applied, the amount of oxidized form of the redox mediator at the counter electrode and the potential difference must be sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the working electrode surface. The diffusion limited current generated by the oxidation of the reduced form of the redox mediator at the working electrode surface is measured by a current measuring meter.

The measured current may be accurately correlated to the concentration of analyte in the sample when the following requirements are satisfied:

- 1) the rate of oxidation of the reduced form of the redox mediator is governed by the rate of diffusion of the reduced form of the redox mediator to the surface of the working electrode; and
- 2) the current produced is limited by the oxidation of the reduced form of the redox mediator at the surface of the working electrode.

In the present inventive device, these requirements are satisfied by employing a readily reversible redox mediator and by supplying reagent 11 with the oxidized form of the redox mediator in an amount sufficient to insure that the current produced during diffusion limited electrooxidation is limited by the oxidation of the

-9-

reduced form of the redox mediator at the working electrode surface. For current produced during electrooxidation to be limited by the oxidation of the reduced form of the redox mediator at the working  
05 electrode surface, the amount of oxidized form of the redox mediator at the surface of the counter electrode must always exceed the amount of the reduced form of the redox mediator at the surface of the working electrode.

10       Importantly, when the reagent includes an excess of the oxidized form of the redox mediator, as described below, the working and counter electrodes may be substantially the same size as well as made of the same electrically conducting material. The ability to utilize  
15 electrodes that are substantially the same size and made of the same material represents an important advantage for manufacturing a biosensor.

A further requirement of the reagent is that the buffer employed must have a higher oxidation potential  
20 than the reduced form of the redox mediator.

The type of enzyme used will depend upon the analyte to be measured. For example, if glucose is the analyte to be measured, glucose oxidase may be used as the enzyme. If cholesterol is the analyte to be measured, cholesterol  
25 oxidase may be used as the enzyme.

As explained above, the redox mediator must be readily reversible and the oxidized form of the redox mediator must be of sufficient type to receive at least one electron from the reaction involving enzyme, analyte, and  
30 oxidized form of the redox mediator. For example, when glucose is the analyte to be measured and glucose oxidase is the enzyme, ferricyanide or quinone may be the oxidized form of the redox mediator.

-10-

Other examples of enzymes and redox mediators (oxidized form) that may be used in measuring particular analytes by the present invention are listed below in Table 1.

-11-

TABLE I

| <u>ANALYTE</u>        | <u>ENZYMES</u>  | <u>REDOX MEDIATOR (OXIDIZED FORM)</u>                                | <u>ADDITIONAL MEDIATOR</u>   |
|-----------------------|---|--|--|
| GLUCOSE               | GLUCOSE DEHYDROGENASE AND<br>DIAPHORASE                                     | FERRICYANIDE   |  |
| CHOLESTEROL           | CHOLESTEROL ESTERASE AND<br>CHOLESTEROL OXIDASE                             | FERRICYANIDE   | 2,6-DIMETHYL-1,4-BENZOQUINONE<br>2,5-DICHLORO-1,4-BENZOQUINONE<br>OR PHENAZINE ETHOSULFATE |
| HDL CHOLESTEROL       | CHOLESTEROL ESTERASE AND<br>CHOLESTEROL OXIDASE                             | FERRICYANIDE   | 2,6-DIMETHYL-1,4-BENZOQUINONE<br>2,5-DICHLORO-1,4-BENZOQUINONE<br>OR PHENAZINE ETHOSULFATE |
| TRIGLYCERIDES         | LIPOPROTEIN LIPASE,<br>GLYCEROL KINASE, AND<br>GLYCEROL-3-PHOSPHATE OXIDASE | FERRICYANIDE OR<br>PHENAZINE ETHOSULFATE                             | PHENAZINE METHOSULFATE   |
| LACTATE               | LACTATE OXIDASE   | FERRICYANIDE   | 2,6-DICHLORO-1,4-BENZOQUINONE  |
| LACTATE               | LACTATE DEHYDROGENASE AND<br>DIAPHORASE                                     | FERRICYANIDE,<br>PHENAZINE ETHOSULFATE, OR<br>PHENAZINE METHOSULFATE |  |
| LACTATE DEHYDROGENASE | DIAPHORASE  | FERRICYANIDE,<br>PHENAZINE ETHOSULFATE, OR<br>PHENAZINE METHOSULFATE |  |
| PYRUVATE              | PYRUVATE OXIDASE  | FERRICYANIDE   |  |
| ALCOHOL               | ALCOHOL OXIDASE   | PHENYLENEDIAMINE   |  |
| BILIRUBIN             | BILIRUBIN OXIDASE   | 1-METHOXY-PHENAZINE METHOSULFATE                                     |  |
| URIC ACID             | URICASE   | FERRICYANIDE   |  |

-12-

In some of the examples shown in Table 1, at least one additional enzyme is used as a reaction catalyst. Also, some of the examples shown in Table 1 may utilize an additional mediator, which facilitates electron transfer to the oxidized form of the redox mediator. The additional mediator may be provided to the reagent in lesser amount than the oxidized form of the redox mediator.

The amount of enzyme included in the reagent may vary depending upon the time period desired for completion of the reaction involving analyte, enzyme, and oxidized form of the redox mediator. The more enzyme added, the shorter the time period for completion of the reaction. When a glucose reagent includes glucose oxidase, more than about 0.5 million international units (units) of glucose oxidase per liter of reagent (referring to the reagent composition before being dried on the electrode surfaces) should be used in the reagent, and preferably about 2 million units of glucose oxidase per liter of reagent is used. Below about 0.5 million units per liter of reagent, assay performance is poor. About 2 million units of glucose oxidase per liter of reagent provides a reagent that will achieve completion of the reaction involving glucose, glucose oxidase, and ferricyanide within about 20 seconds, a conveniently short time period for the reaction. Above about 2 million units of glucose oxidase per liter of reagent, the reagent is needlessly more expensive to produce. (These amounts of glucose oxidase refer to the reagent composition before it is dried onto the electrode surfaces.)

The actual amount of oxidized form of the redox mediator needed in the reagent is governed by the concentration range of the analyte sought to be measured. The reagent for analyzing glucose (described herein) includes sufficient redox mediator (oxidized form) to

-13-

measure the glucose level in a sample of human whole blood from about 10 to about 70 microliters ( $\mu$ l) in volume. The reagent must be provided with enough of the oxidized form of the redox mediator so that when the potential  
05 difference is applied between electrodes 4 and 5, the amount of oxidized form of the redox mediator at the surface of the counter electrode exceeds the amount of reduced form of the redox mediator at the working electrode.

10 The upper limit of the amount of redox mediator (oxidized form) will usually depend on the mediator's solubility and dispersion properties in the reagent. The reagent for the present invention, exemplified by a biosensor for the assay of glucose, preferably includes a  
15 microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent.

Examples of microcrystalline materials that will disperse the redox mediator are microcrystalline  
20 cellulose, dextrans, and chitin. The amount of microcrystalline material included in the preferred glucose reagent, which includes glucose oxidase and potassium ferricyanide, is from about 1% (weight:volume) to about 4.5% (weight:volume), and is preferably about  
25 1.5% (weight:volume). Below about 1% (weight:volume) microcrystalline material, the reagent will fall off of the electrode surfaces after drying. Above about 4.5% (weight:volume) microcrystalline material, the reagent gels. For a glucose reagent that includes ferricyanide  
30 and glucose oxidase, the preferred microcrystalline material is a combination of AVICEL RC-591F (a microcrystalline cellulose available from FMC Corp.) and NATROSOL-250 M (a micro-crystalline carboxymethylcellulose available from Aqualon). The amount of AVICEL in the  
35 reagent may range to from about 1% to about 4.2 % (weight:volume) and is preferably about 1.4%

-14-

(weight:volume). The amount of NATROSOL in the reagent may range from about 0% to about 0.3% (weight:volume) and is preferably about 0.06% (weight:volume). (These percentages refer to the reagent composition before it is dried onto the electrode surfaces.)

05        When AVICEL and NATROSOL are added to the reagent, in the ranges stated above, the amount of potassium ferricyanide that may be incorporated into the reagent may range from about 0.15 molar (M) to about 0.7 M, and is preferably about 0.3 M. When the concentration of  
10        ferricyanide is below about 0.15 M and above about 0.7 M, performance of the biosensor decreases. (These molarities refer to the reagent composition before it is dried onto the electrode surfaces.)

15        The reagent also preferably includes a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte to be measured. For example, in a reagent for analyzing a sample of human whole blood containing glucose, the surfactant is preferably a nonionic surfactant. From about 0% (weight:volume) to  
20        about 0.3% (weight:volume) surfactant may be present in the reagent. Above about 0.3% (weight:volume) surfactant, red blood cells begin to hemolyze. The preferred surfactant in the glucose reagent is TRITON X-100 (available from Sigma Chemical Corporation) at a preferred  
25        concentration of about 0.05% (weight:volume.). (Percentages refer to the reagent composition before it is dried onto the electrode surfaces.)

30        Any buffer that satisfies the above-stated requirements of providing a satisfactory pH for enzyme function and having a higher oxidation potential than the reduced form of the redox mediator may be used.

-15-

Examples of such buffers for a glucose reagent utilizing the enzyme glucose oxidase include phosphate, citrate (citrate also helps stabilize the reagent), "Good" buffers (e.g., 2-(N-morpholino) ethanesulfonic acid, N-(2-acetamido) iminodiacetic acid, piperazine- N,N-bis (2-ethanesulfonic acid), N-(2- acetamido)-2-aminoethanesulfonic acid, N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid, N- tris (hydroxymethyl) -methyl-2-aminoethanesulfonic acid, and N-2- hydroxyethyl-piperazine - N - 2- ethanesulfonic acid, and Tris buffers (buffers derived from 2-amino- 2 (hydroxymethyl) -1,3 -propanediol). ("Good" and Tris buffers are available from Sigma Chemical Company.) Imidazole should not be used as a buffer. These buffers may be used to provide a preferred pH range from about 4 to about 8. The most preferred pH range is from about 6 to about 7. The most preferred buffer is phosphate (e.g., potassium phosphate) from about 0.1 M to about 0.5 M and preferably about 0.4 M. (These concentration ranges refer to the reagent composition before it is dried onto the electrode surfaces.)

The reagent preferably further includes a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent. The reagent stabilizer stabilizes the enzyme, and for a glucose reagent containing glucose oxidase, the reagent stabilizer may be selected from a group consisting of glutamate, aspartate, blue dextran and trehalose. The preferred reagent stabilizer for a reagent containing glucose oxidase is glutamate (e.g., potassium glutamate) at a concentration range from about 0 % (weight:volume) to about 4% (weight:volume), with the preferred amount being about 2% (weight:volume). (These percentages refer to the reagent composition before it is dried onto the electrode surfaces.)

-16-

A protocol for making the preferred glucose reagent utilizing the enzyme glucose oxidase and ferricyanide as the oxidized form of the redox mediator is as follows:

- 05 Step 1- Prepare 1 liter (in a volumetric flask) of a buffer/NATROSOL mixture by adding 1.2000 grams (g) NATROSOL-250 M to 0.740 M aqueous potassium phosphate buffer (including 80.062 g monobasic potassium phosphate and 26.423 g dibasic potassium phosphate) at pH 6.25. Allow the NATROSOL to stir and swell for 3 hours.
- 10 Step 2- Prepare an AVICEL mixture by stirring 14.0000 g AVICEL RC-591 F and 504.7750 g water for 20 minutes.
- Step 3- Prepare a TRITON mixture by adding 0.5000 g TRITON X-100 to 514.6000 g of the buffer/NATROSOL mixture and stir for 15 minutes.
- 15 Step 4- While stirring, add the total TRITON mixture dropwise with a dropwise addition funnel or buret to the total AVICEL mixture. Once addition is complete, continue stirring overnight.
- 20 Step 5- To the mixture resulting from Step 4, add, while stirring, 98.7750 g potassium ferricyanide. (Add a little potassium ferricyanide at a time to allow the potassium ferricyanide to dissolve as added.)
- Step 6- Stir the resulting mixture of Step 5 for 20 minutes.
- 25 Step 7- Adjust the pH of the mixture resulting from Step 6 to 6.25 by adding potassium hydroxide.
- Step 8- To the resulting mixture of Step 6 add 9.1533 g glucose oxidase (218.50 units per milligram (mg) from Biozyme) and stir at least 20 minutes.

-17-

Step 9- To the resulting mixture of Step 8 add 20 g potassium glutamate and stir at least 20 minutes.

Step 10- Filter the resulting mixture of Step 9 through a 100 micron sieve bag to remove any AVICEL clumping. The  
05 filtrate is the resulting reagent composition, which is added to the electrode surfaces and is then dried.

In the preferred embodiment for glucose determination, 6 microliters ( $\mu$ l) of reagent made by the above-stated protocol is added to well 9 formed by cutout 8. This  
10 amount of reagent 11 will substantially cover surface areas 10 on both electrodes (Figs. 1 and 2) and will also contain a sufficient amount of ferricyanide, and a sufficient amount of enzyme (glucose oxidase) to catalyze the oxidation of glucose (from a sample of human whole  
15 blood) and the reduction of ferricyanide to completion within about 20 seconds.

Reagent 11 is then dried by heating at about 50° C for about 3 minutes. Drying removes at least about 90% of the water content of the reagent, thereby resulting in a  
20 preferred dried reagent with the following proportions of components: from about 1.1 to about 1.5 millimoles (m mol) ferricyanide per gram of dried reagent; from about 2,300 to about 2,600 units of glucose oxidase per gram of dried reagent, assuming 75% loss of enzyme activity (an  
25 unusually high loss of enzyme activity) from reagent drying, from about 8,600 to about 9,600 units of glucose oxidase per gram of dried reagent, assuming a more typical 6% loss of enzyme activity from reagent drying, and from about 9,200 to about 10,400 units of glucose oxidase per  
30 gram of dried reagent, assuming no loss of enzyme activity from reagent drying; from about 1.3 to about 1.9 m mol phosphate buffer per gram of dried reagent; from about 2 to about 3 milligrams (mg) NATROSOL-250 M per gram of dried reagent and from about 50 to about 71 mg AVICEL

-18-

RC-591 F per gram of dried reagent (a total of from about 52 to about 74 mg microcrystalline material per gram of dried reagent); from about 71 to about 102 mg glutamate per gram of dried reagent; and from about 2 to about 3 mg  
05 TRITON X-100 per gram of dried reagent.

As stated above, each component of the formulated reagent (before drying) may vary within the disclosed limits. Therefore, a glucose reagent dried as above may include the following broader ranges of components: from  
10 about 0.55 to about 3.5 m mol ferricyanide per gram of dried reagent; more than about 570 units of glucose oxidase per gram of dried reagent, assuming a 75% loss of enzyme activity (an unusually high loss of enzyme  
15 units of glucose oxidase per gram of dried reagent, assuming a more typical 6% loss of enzyme activity from reagent drying; from about 0.35 to about 2.6 m mol phosphate per gram of dried reagent; from about 0 to about 15 mg NATROSOL-250 M per gram of dried reagent and from  
20 about 36 to about 213 mg AVICEL RC-591 F per gram of dried reagent ( a total of from about 36 to about 228 mg microcrystalline material per gram of dried reagent); from about 0 to about 200 mg glutamate per gram of dried reagent; and from about 0 to about 18 mg TRITON X-100 per  
25 gram of dried reagent.

After drying, a polyester or nylon mesh 13 (Figs. 2 and 3) is preferably placed on top of the dried reagent to aid in preventing loss of reagent from the biosensor during shipping and handling and to aid in minimizing  
30 human contamination from the reagent. Mesh 13 is affixed to the inventive device by adhesive tape 14, which includes hole 15. (Figs 2 and 3) Hole 15 is the target area for adding a sample containing an analyte to be measured by the inventive device. (Fig. 3)

-19-

After drying the reagent and affixing the mesh, the roll-formed biosensors are separated by die punching to form discrete biosensors, which are used in conjunction with 1) a power source in electrical connection with the  
05 working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode, and 2) a meter in  
10 electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by oxidation of the reduced form of the redox mediator when the above-stated electrical potential difference is applied.

15 The meter described above will normally be adapted to apply an algorithm to the current measurement, whereby an analyte concentration is provided and visually displayed. Improvements in such a power source and meter are the subject of commonly assigned U.S. Patent Number 4,963,814  
20 (issued October 16, 1990), and U.S. Patent Application Serial Numbers 07/451,212 (filed December 15, 1989; Notice of Allowance issued November 6, 1990; Issue Fee Paid November 30, 1990), 07/451,108 (filed December 15, 1989; Notice of Allowance issued September 24, 1990; Issue Fee  
25 Paid October 31, 1990), and 07/451,309 (filed December 15, 1989), the disclosures of which are hereby incorporated by reference.

For easy electrical connection of the power source and meter, additional cutout portion 12 (Figs. 1 through 3),  
30 exposing portions of the working and counter electrodes, are preferably provided in the biosensor device.

The biosensor device described above may be used to determine the concentration of an analyte in a fluid sample by performing the following steps:

-20-

- a) contacting the fluid sample with a reagent (described above) that substantially covers substantially equal surface areas of working and counter electrodes;
- b) allowing the reaction between the analyte and the oxidized form of the redox mediator to go to completion;
- c) subsequently applying a potential difference between the electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode;
- d) thereafter measuring the resulting diffusion limited current; and
- e) correlating the current measurement to the concentration of analyte in the fluid.

Many analyte-containing fluids may be analyzed. For example, analytes in human body fluids such as whole blood, blood serum, urine and cerebrospinal fluid may be measured. Also, analytes found in fermentation products and in environmental substances, which potentially contain environmental contaminants, may be measured.

When measuring analytes found in human body fluids, especially whole blood, the potential difference applied between the electrodes should be no more than about 500 millivolts. When a potential difference above about 500 millivolts is applied between the electrodes, oxidation of the working electrode surface (for palladium) and of some blood components can become intolerable, thereby preventing an accurate and precise correlation of current to analyte concentration. For an assay of glucose in a whole blood sample, wherein the oxidized form of the redox mediator is ferricyanide, a potential difference from about 150 millivolts to about 500 millivolts may be

-21-

applied between the electrodes to achieve diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode. Preferably, about 300 millivolts potential difference is applied between the electrodes.

05

Current generated from the oxidation of the reduced form of the redox mediator may be measured at any time from about 0.5 seconds to about 30 seconds after the potential difference is applied between the electrodes.

10

At less than about 0.5 seconds, diffusion limited current has not been achieved. After about 30 seconds, convection becomes significant, thereby interfering with the measurement of a diffusion limited current. Preferably, current is measured at about 10 seconds after the potential difference is applied between the electrodes, and the measured current is correlated to the concentration of analyte in the sample.

15

In the preferred method for analysis of glucose from a sample of human whole blood, 20  $\mu$ l of whole blood is added to the above-stated preferred glucose reagent. The reaction of glucose and ferricyanide is allowed to go to completion, thereby forming gluconic acid and ferrocyanide. This reaction normally requires a short time to go to completion, and in the preferred embodiment, the reaction goes to completion in less than about 20 seconds. About twenty seconds after addition of the whole blood sample, a potential difference of about 300 millivolts is applied between the electrodes, thereby oxidizing ferrocyanide to ferricyanide at the surface of the working electrode. About 10 seconds after the potential difference is applied to the electrodes, current is measured and correlated to the concentration of glucose in the blood sample.

20

25

30

-22-

The glucose concentration of a sample may be accurately and precisely measured by the present inventive method utilizing the present inventive biosensor. Further, when a sample of human whole blood is measured, error due to hematocrit effect is insignificant.

As a variation of the present invention, the counter electrode could be smaller than the working electrode. When the counter electrode is smaller than the working electrode, the amount of oxidized form of the redox mediator supplied to reagent 11 must be increased. The amount of oxidized form of the redox mediator in reagent 11 must be increased because the above-stated requirements for accurately correlating concentration of the analyte to current must be satisfied; that is,

1) the rate of oxidation of the reduced form of the redox mediator is governed by the rate of diffusion of the reduced form of the redox mediator to the surface of the working electrode; and

2) the current produced is limited by the oxidation of the reduced form of the redox mediator at the surface of the working electrode.

For example, it has been shown that when the counter electrode is about half the size of the working electrode, a mixture of about 2700 nanomoles (nmol) of ferricyanide and about 900 nmol of ferrocyanide (dissolved in 20  $\mu$ l of water) satisfied the requirements stated above.

Also, the present invention has been illustrated by analytes that are oxidized and redox mediators that are reduced in the presence of a catalytic amount of enzyme. However, the present inventive device, reagents and methods may also be used to measure the concentration of an analyte in a fluid sample, wherein the analyte is

-23-

reduced and the reduced form of a redox mediator is oxidized in the presence of a catalytic amount of an enzyme (e.g., a reductase). After the reaction involving analyte, enzyme, and reduced form of the redox mediator reaches completion, a potential difference is applied between the electrodes. The amount of reduced form of the redox mediator at the counter electrode (in this case an anode rather than a cathode) and the applied potential difference must be sufficient to cause diffusion limited electroreduction of the oxidized form of the redox mediator at the surface of the working electrode (in this case a cathode rather than an anode). The diffusion limited current generated by reduction of the oxidized form of the the redox mediator at the working electrode surface is correlated to the concentration of analyte in the sample being analyzed.

The redox mediator must be readily reversible and the amount of reduced form of the redox mediator in reagent 11 must be sufficient to insure that the current produced during electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface.

The buffer must also have a lower reduction potential than the reduction potential of the oxidized form of the redox mediator and must be of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving analyte, enzyme and the reduced form of the redox mediator. These and other requirements are analogous to those for measuring an analyte that is oxidized rather than reduced.

The present invention has been disclosed in the above teachings and drawings with sufficient clarity and conciseness to enable one skilled in the art to make and use the invention, to know the best mode for carrying out

-24-

the invention, and to distinguish it from other inventions and from what is old. Many variations and obvious adaptations of the invention will readily come to mind, and these are intended to be contained within the scope of the invention as claimed below.

05

-25-

What is claimed is:

1. A device for analyzing an analyte, comprising:

a. a first electrical insulator;

05 b. working and counter electrodes of substantially the same size, the electrodes being made of the same electrically conducting materials and being supported on the first electrical insulator;

10 c. a second electrical insulator, overlaying the first electrical insulator and the electrodes and including a cutout portion that exposes substantially equal surface areas of the working and counter electrodes; and

15 d. a reagent, substantially covering the exposed electrode surfaces in the cutout portion and comprising the oxidized form of a redox mediator, an enzyme, and a buffer,

20 the oxidized form of the redox mediator being of sufficient type to receive at least one electron from a reaction involving enzyme, analyte, and oxidized form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electrooxidation is limited by the oxidation of the reduced form of the redox mediator at the working electrode surface,

25 the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and oxidized form of the redox mediator, and

the buffer having a higher oxidation potential than the reduced form of the redox mediator and being of

-26-

sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and oxidized form of the redox mediator.

- 05 2. The device of claim 1, wherein the reagent further comprises a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent.
- 10 3. The device of claim 1, wherein the reagent further comprises at least one additional enzyme and an additional redox mediator.
- 15 4. The device of claim 1, wherein the electrically conducting material of the working and counter electrodes is selected from a group consisting of palladium, platinum, gold, silver, titanium, copper, and carbon.
5. The device of claim 2, wherein the reagent further comprises a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte.
- 20 6. The device of claim 5, wherein the reagent further comprises a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.
7. The device of claim 1, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, and the enzyme is glucose oxidase.
- 25 8. The device of claim 2, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide and the enzyme is glucose oxidase.
9. The device of claim 8, wherein the amount of ferricyanide is from about 0.55 to about 3.5 millimoles

-27-

per gram of reagent.

10. The device of claim 6, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, the buffer is phosphate, the  
05 microcrystalline material includes microcrystalline cellulose and microcrystalline carboxymethylcellulose, the enzyme is glucose oxidase, the surfactant is a nonionic surfactant and the reagent stabilizer is selected from a group consisting of glutamate, aspartate, blue dextran,  
10 and trehalose.

11. The device of claim 10, wherein the amount of ferricyanide is from about 0.55 to about 3.5 millimoles per gram of reagent, the amount of phosphate buffer is from about 0.35 to about 2.6 millimoles per gram of  
15 reagent, the amount of microcrystalline material is from about 36 to about 228 milligrams per gram of reagent, the amount of glucose oxidase is more than about 570 units per gram of reagent, the amount of surfactant is from about 0 to about 18 milligrams per gram of reagent, and the amount  
20 of reagent stabilizer is from about 0 to about 200 milligrams per gram of reagent.

12. A device for analyzing glucose, comprising:

- a. a first electrical insulator;
- b. working and counter electrodes of substantially  
25 the same size, the electrodes being made of palladium and being supported on the first electrical insulator;
- c. a second electrical insulator overlaying the first electrical insulator and the electrodes and including a cutout portion that exposes substantially equal surface  
30 areas of the working and counter electrodes; and

-28-

d. a reagent, substantially covering the exposed electrode surfaces in the cutout portion and comprising

1) from about 1.1 to about 1.5 millimoles ferricyanide per gram of reagent,

05 2) from about 1.3 to about 1.9 millimoles phosphate buffer per gram of reagent,

3) from about 2300 to about 10,400 units of glucose oxidase per gram of reagent,

10 4) from about 50 to about 71 milligrams microcrystalline cellulose per gram of reagent,

5) from about 2 to about 3 milligrams microcrystalline carboxymethylcellulose per gram of reagent,

15 6) from about 2 to about 3 milligrams TRITON X-100 per gram of reagent, and

7) from about 71 to about 102 milligrams glutamate per gram of reagent.

13. The device of claim 1, further comprising:

20 e. a power source in electrical connection with the working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode; and

25 f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the oxidation of the

-29-

reduced form of the redox mediator at the working electrode surface.

14. The device of claim 7, further comprising:

05 e. a power source in electrical connection with the working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode; and

10 f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the oxidation of the reduced form of the redox mediator at the working electrode surface.

15 15. The device of claim 12, wherein the second electrical insulator further includes an additional cutout portion exposing portions of the working and counter electrodes, and the device further comprises:

20 e. a power source in electrical connection with the working and counter electrodes at the additional cutout portion, the power source being capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator  
25 at the surface of the working electrode; and

f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the oxidation of the reduced form of the redox mediator at the working  
30 electrode surface.

-30-

16. A reagent for an electrochemical device that has working and counter electrodes and measures an analyte, comprising:

05       the oxidized form of a redox mediator, an enzyme, and  
a buffer,

10       the oxidized form of the redox mediator being of  
sufficient type to receive at least one electron from a  
reaction involving enzyme, analyte, and oxidized form of  
the redox mediator and being in sufficient amount to  
insure that current produced by diffusion limited  
electrooxidation is limited by the oxidation of the  
reduced form of the redox mediator at the working  
electrode surface,

15       the enzyme being of sufficient type and in sufficient  
amount to catalyze the reaction involving enzyme, analyte,  
and oxidized form of the redox mediator, and

20       the buffer having a higher oxidation potential than  
the reduced form of the redox mediator and being of  
sufficient type and in sufficient amount to provide and  
maintain a pH at which the enzyme catalyzes the reaction  
involving enzyme, analyte, and oxidized form of the redox  
mediator.

25       17. The reagent of claim 16, wherein the reagent further  
comprises at least one additional enzyme and an additional  
redox mediator.

18. The reagent of claim 16, further comprising:

      a microcrystalline material of sufficient type and in  
sufficient amount to disperse the redox mediator in the  
reagent.

-31-

19. The reagent of claim 18, further comprising:

a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte.

20. The reagent of claim 19, further comprising:

05 a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

21. The reagent of claim 20, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, the buffer is phosphate, the  
10 microcrystalline material includes microcrystalline cellulose and microcrystalline carboxymethylcellulose, the enzyme is glucose oxidase, the surfactant is a nonionic surfactant, and the reagent stabilizer is selected from a group consisting of glutamate, aspartate, blue dextran,  
15 and trehalose.

22. The reagent of claim 21, wherein the amount of ferricyanide is from about 0.55 to about 3.5 millimoles per gram of reagent, the amount of phosphate buffer is from about 0.35 to about 2.6 millimoles per gram of  
20 reagent, the amount of microcrystalline material is from about 36 to about 228 milligrams per gram of reagent, the amount of glucose oxidase is more than about 570 units per gram of reagent, the amount of surfactant is from about 0 to about 18 milligrams per gram of reagent, and the amount  
25 of reagent stabilizer is from about 0 to about 200 milligrams per gram of reagent.

23. A reagent for an electrochemical device that has working and counter electrodes and measures glucose, comprising:

-32-

- a. from about 1.1 to about 1.5 millimoles ferricyanide per gram of reagent;
  - b. from about 1.3 to about 1.9 millimoles phosphate buffer per gram of reagent;
  - 05 c. from about 2300 to about 10,400 units of glucose oxidase per gram of reagent;
  - d. from about 50 to about 71 milligrams microcrystalline cellulose per gram of reagent;
  - 10 e. from about 2 to about 3 milligrams microcrystalline carboxymethylcellulose per gram of reagent;
  - f. from about 2 to about 3 milligrams TRITON X-100 per gram of reagent; and
  - g. from about 71 to about 102 milligrams glutamate per gram of reagent.
- 15 24. A method of determining the concentration of an analyte in a fluid, comprising the steps of:
- a. contacting the fluid with a reagent that covers substantially equal surface areas of working and counter electrodes and includes the oxidized form of a redox mediator, an enzyme, and a buffer,
  - 20
- 25 the oxidized form of the redox mediator being of sufficient type to receive at least one electron from a reaction involving enzyme, analyte, and oxidized form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electrooxidation is limited by the oxidation of the reduced form of the redox mediator at the working electrode surface,

-33-

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the oxidized form of the redox mediator, and

05 the buffer having a higher oxidation potential than the reduced form of the redox mediator and being of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the oxidized form of the redox mediator;

10 b. allowing the reaction involving the enzyme, analyte, and the oxidized form of the redox mediator to go to completion;

c. subsequently applying a potential difference between the electrodes sufficient to cause diffusion  
15 limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode;

d. thereafter measuring the resulting diffusion limited current; and

e. correlating the current measurement to the  
20 concentration of the analyte in the fluid.

25. The method of claim 24, wherein the reagent further includes at least one additional enzyme and an additional redox mediator.

26. The method of claim 24, wherein the reagent further  
25 includes a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent.

27. The method of claim 26, wherein the reagent further includes

-34-

a surfactant of sufficient type and in sufficient amount to wet the fluid upon contact with the reagent, and

a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

05 28. The method of claim 27, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, the buffer is phosphate, the microcrystalline material includes microcrystalline  
10 cellulose and microcrystalline carboxymethylcellulose, the enzyme is glucose oxidase, the surfactant is a nonionic surfactant, and the reagent stabilizer is selected from a group consisting of glutamate, aspartate, blue dextran, and trehalose.

15 29. The reagent of claim 28, wherein the amount of ferricyanide is from about 0.55 to about 3.5 millimoles per gram of reagent, the amount of phosphate buffer is from about 0.35 to about 2.6 millimoles per gram of reagent, the amount of microcrystalline material is from about 36 to about 228 milligrams per gram of reagent, the  
20 amount of glucose oxidase is more than about 570 units per gram of reagent, the amount of surfactant is from about 0 to about 18 milligrams per gram of reagent, and the amount of reagent stabilizer is from about 0 to about 200 milligrams per gram of reagent.

25 30. A method of determining the concentration of glucose in a fluid, comprising the steps of:

a. contacting the fluid with a reagent that covers substantially equal surface areas of working and counter electrodes and includes

30 from about 1.1 to about 1.5 millimoles ferricyanide per gram of reagent,

-35-

from about 1.3 to about 1.9 millimoles phosphate buffer per gram of reagent,

from about 2300 to about 10,400 units of glucose oxidase per gram of reagent,

05 from about 50 to 71 milligrams microcrystalline cellulose per gram of reagent,

from about 2 to about 3 milligrams microcrystalline carboxymethylcellulose per gram of reagent, and

10 from about 71 to about 102 milligrams glutamate per gram of reagent;

b. allowing the reaction involving the enzyme, analyte, and the oxidized form of the redox mediator to go to completion;

15 c. subsequently applying a potential difference between the electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode;

d. thereafter measuring the resulting diffusion limited current; and

20 e. correlating the current measurement to the concentration of glucose in the fluid.

31. A device for analyzing an analyte, comprising:

a. a first electrical insulator;

25 b. working and counter electrodes of substantially the same size, the electrodes being made of the same

-36-

electrically conducting materials and being supported on the first electrical insulator;

05 c. a second electrical insulator, overlaying the first electrical insulator and the electrodes and including a cutout portion that exposes substantially equal surface areas of the working and counter electrodes; and

10 d. a reagent, substantially covering the exposed electrode surfaces in the cutout portion and comprising the reduced form of a redox mediator, an enzyme, and a buffer,

15 the reduced form of the redox mediator being of sufficient type to donate at least one electron from a reaction involving enzyme, analyte, and reduced form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface,

20 the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the reduced form of the redox mediator, and

25 the buffer having a lower reduction potential than the oxidized form of the redox mediator and being of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the reduced form of the redox mediator.

32. The device of claim 31, wherein the reagent further comprises

-37-

a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent,

05 a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte, and

a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

33. The device of claim 31, further comprising:

10 e. a power source in electrical connection with the working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electroreduction of the oxidized form of the redox mediator at the surface of the working electrode; and

15 f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the reduction of the oxidized form of the redox mediator at the working electrode surface.

20 34. A reagent for an electrochemical device that has working and counter electrodes and measures an analyte, comprising:

the reduced form of a redox mediator, an enzyme, and buffer,

25 the reduced form of the redox mediator being of sufficient type to donate at least one electron from a reaction involving enzyme, analyte, and reduced form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited

-38-

electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface,

05 the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the reduced form of the redox mediator, and

10 the buffer having a lower reduction potential than the oxidized form of the redox mediator and being of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the reduced form of the redox mediator.

35. The reagent of claim 34, further comprising:

15 a microcrystalline material of sufficient type and in sufficient amount of disperse the redox mediator in the reagent;

a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte; and

20 a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

36. A method of determining the concentration of an analyte in a fluid, comprising the steps of:

25 a. contacting the fluid with a reagent that covers substantially equal surface areas of working and counter electrodes and includes the reduced form of a redox mediator, an enzyme, and a buffer,

30 the reduced form of the redox mediator being of sufficient type to donate at least one electron from a reaction involving enzyme, analyte, and reduced form of the redox mediator and being in sufficient amount to

-39-

insure that current produced by diffusion limited electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface,

05       the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the reduced form of the redox mediator, and

          the buffer having lower reduction potential than the oxidized form of the redox mediator and being of  
10       sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the reduced form of the redox mediator;

          b. allowing the reaction involving the enzyme,  
15       analyte, and the reduced form of a redox mediator to go to completion;

          c. subsequently applying a potential difference between the electrodes sufficient to cause diffusion limited electroreduction of the oxidized form of the redox  
20       mediator at the surface of the working electrode;

          d. thereafter measuring the resulting diffusion limited current; and

          e. correlating the current measurement to the concentration of the analyte in the fluid.

25       37. The method of claim 36, wherein the reagent further includes

          a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the

-40-

reagent,

a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte, and

05 a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

38. A device for analyzing an analyte, comprising:

a. a first electrical insulator;

10 b. a working electrode and a counter electrode that is smaller than the working electrode, the electrodes being made of the same electrically conducting materials and being supported on the first electrical insulator;

15 c. a second electrical insulator, overlaying the first electrical insulator and the electrodes and including a cutout portion that exposes a smaller surface area of the counter electrode than the working electrode; and

20 d. a reagent, substantially covering the exposed electrode surfaces in the cutout portion and comprising the oxidized form of a redox mediator, an enzyme, and a buffer,

25 the oxidized form of the redox mediator being of sufficient type to receive at least one electron from a reaction involving enzyme, analyte, and oxidized form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electrooxidation is limited by the oxidation of the reduced form of the redox mediator at the working electrode surface,

-41-

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and oxidized form of the redox mediator, and

05 the buffer having a higher oxidation potential than the reduced form of the redox mediator and being of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and oxidized form of the redox mediator.

10 39. The device of claim 38, wherein the reagent further comprises a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent.

15 40. The device of claim 38, wherein the reagent further comprises at least one additional enzyme and an additional redox mediator.

20 41. The device of claim 38, wherein the electrically conducting material of the working and counter electrodes is selected from a group consisting of palladium, platinum, gold, silver, titanium, copper, and carbon.

42. The device of claim 39, wherein the reagent further comprises a surfactant of sufficient type and in sufficient amount to wet a sample containing the analyte.

25 43. The device of claim 42, wherein the reagent further comprises a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

44. The device of claim 38, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, and the enzyme is glucose oxidase.

-42-

45. The device of claim 39, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide and the enzyme is glucose oxidase.

05 46. The device of claim 43, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, the buffer is phosphate, the microcrystalline material includes microcrystalline cellulose and microcrystalline carboxymethylcellulose, the enzyme is glucose oxidase, the surfactant is a nonionic  
10 surfactant and the reagent stabilizer is selected from a group consisting of glutamate, aspartate, blue dextran, and trehalose.

47. The device of claim 38, further comprising:

15 e. a power source in electrical connection with the working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode; and

20 f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the oxidation of the reduced form of the redox mediator at the working electrode surface.

25 48. A method of determining the concentration of an analyte in a fluid, comprising the steps of:

30 a. contacting the fluid with a reagent that covers a smaller surface area of a counter electrode than a working electrode and includes the oxidized form of a redox mediator, an enzyme, and a buffer,

-43-

the oxidized form of the redox mediator being of sufficient type to receive at least one electron from a reaction involving enzyme, analyte, and oxidized form of the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electrooxidation is limited by the oxidation of the reduced form of the redox mediator at the working electrode surface,

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the oxidized form of the redox mediator, and

the buffer having a higher oxidation potential than the reduced form of the redox mediator and being of sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the oxidized form of the redox mediator;

b. allowing the reaction involving the enzyme, analyte, and the oxidized form of the redox mediator to go to completion;

c. subsequently applying a potential difference between the electrodes sufficient to cause diffusion limited electrooxidation of the reduced form of the redox mediator at the surface of the working electrode;

d. thereafter measuring the resulting diffusion limited current; and

e. correlating the current measurement to the concentration of the analyte in the fluid.

49. The method of claim 48, wherein the reagent further includes at least one additional enzyme and an additional

-44-

redox mediator.

05 50. The method of claim 48, wherein the reagent further includes a microcrystalline material of sufficient type and, in sufficient amount to disperse the redox mediator in the reagent.

51. The method of claim 50, wherein the reagent further includes

a surfactant of sufficient type and in sufficient amount to wet the fluid upon contact with the reagent, and

10 a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

15 52. The method of claim 51, wherein the analyte is glucose, the oxidized form of the redox mediator is ferricyanide, the buffer is phosphate, the microcrystalline material includes microcrystalline cellulose and microcrystalline carboxymethylcellulose, the enzyme is glucose oxidase, the surfactant is a nonionic surfactant, and the reagent stabilizer is selected from a group consisting of glutamate, aspartate, blue dextran, and trehalose.

20

53. A device for analyzing an analyte, comprising:

a. a first electrical insulator;

25 b. a working electrode and a counter electrode that is smaller than the working electrode, the electrodes being made of the same electrically conducting materials and being supported on the first electrical insulator;

c. a second electrical insulator, overlaying the first electrical insulator and the electrodes and including a

-45-

cutout portion that exposes a smaller surface area of the counter electrode than the working electrode; and

d. a reagent, substantially covering the exposed electrode surfaces in the cutout portion and comprising  
05 the reduced form of a redox mediator, an enzyme, and a buffer,

the reduced form of the redox mediator being of sufficient type to donate at least one electron from a reaction involving enzyme, analyte, and reduced form of  
10 the redox mediator and being in sufficient amount to insure that current produced by diffusion limited electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface,

15 the enzyme being of sufficient type and in sufficient amount to catalyze the reaction involving enzyme, analyte, and the reduced form of the redox mediator, and

the buffer having a lower reduction potential than the oxidized form of the redox mediator and being of  
20 sufficient type and in sufficient amount to provide and maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the reduced form of the redox mediator.

54. The device of claim 53, wherein the reagent further  
25 comprises

a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent,

a surfactant of sufficient type and in sufficient  
30 amount to wet a sample containing the analyte, and

-46-

a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

55. The device of claim 53, further comprising:

05 e. a power source in electrical connection with the working and counter electrodes and capable of supplying an electrical potential difference between the working and counter electrodes sufficient to cause diffusion limited electroreduction of the oxidized form of the redox mediator at the surface of the working electrode; and

10 f. a meter in electrical connection with the working and counter electrodes and capable of measuring the diffusion limited current produced by the reduction of the oxidized form of the redox mediator at the working electrode surface.

15 56. A method of determining the concentration of an analyte in a fluid, comprising the steps of:

a. contacting the fluid with a reagent that covers a smaller surface area of a counter electrode than a working electrode and includes the reduced form of a redox mediator, an enzyme, and a buffer,

20

the reduced form of the redox mediator being of sufficient type to donate at least one electron from a reaction involving enzyme, analyte, and reduced form of the redox mediator and being in sufficient amount to

25 insure that current produced by diffusion limited electroreduction is limited by the reduction of the oxidized form of the redox mediator at the working electrode surface,

the enzyme being of sufficient type and in sufficient

30 amount to catalyze the reaction involving enzyme, analyte,

-47-

and the reduced form of the redox mediator, and

the buffer having lower reduction potential than the oxidized form of the redox mediator and being of sufficient type and in sufficient amount to provide and  
05 maintain a pH at which the enzyme catalyzes the reaction involving enzyme, analyte, and the reduced form of the redox mediator;

b. allowing the reaction involving the enzyme, analyte, and the reduced form of the redox mediator to go  
10 to completion;

c. subsequently applying a potential difference between the electrodes sufficient to cause diffusion limited electroreduction of the oxidized form of the redox mediator at the surface of the working electrode;

15 d. thereafter measuring the resulting diffusion limited current; and

e. correlating the current measurement to the concentration of the analyte in the fluid.

57. The method of claim 56, wherein the reagent further  
20 includes

a microcrystalline material of sufficient type and in sufficient amount to disperse the redox mediator in the reagent,

a surfactant of sufficient type and in sufficient  
25 amount to wet the fluid upon contact with the reagent, and

a reagent stabilizer of sufficient type and in sufficient amount to stabilize the reagent.

1/2

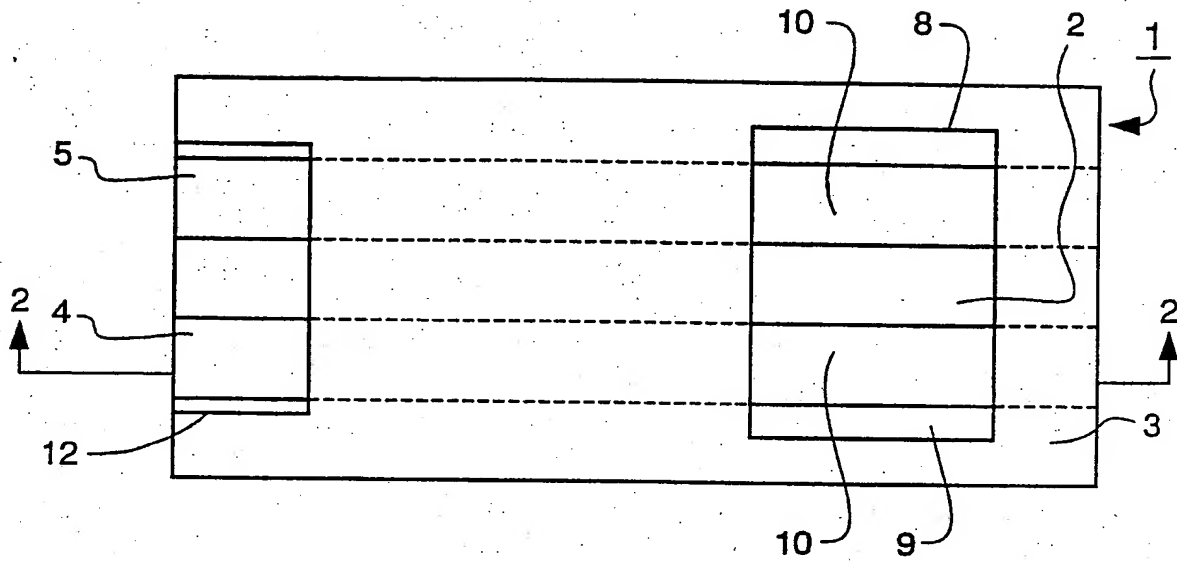


FIG. 1

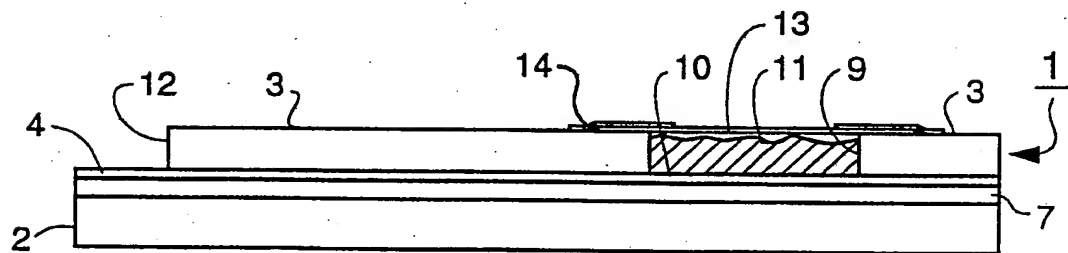
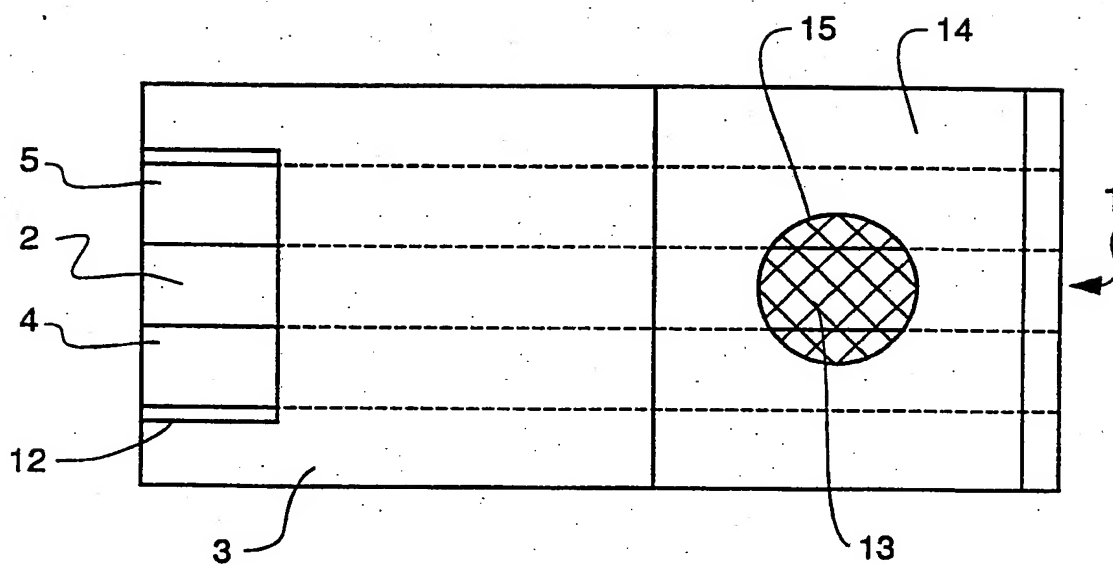


FIG. 2

2/2



**FIG. 3**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/07374

|   |  |   |                         |                        |                              |  |
|---|--|---|-------------------------|------------------------|------------------------------|--|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup><br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC(5) C12Q 1/54; C12M 1/36<br>U.S. CL.: 435/14,288,817   |  |   |                         |                        |                              |  |
| <b>II. FIELDS SEARCHED</b><br><div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>4</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System :</td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; height: 40px; vertical-align: top;">U.S. CL.      435/14,288,817</td> <td style="border: none;"></td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation<br/>to the Extent that such Documents are Included in the Fields Searched <sup>5</sup></div>  |  |   | Classification System : | Classification Symbols | U.S. CL.      435/14,288,817 |  |
| Classification System :   | Classification Symbols   |   |                         |                        |                              |  |
| U.S. CL.      435/14,288,817  |  |   |                         |                        |                              |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>   |  |   |                         |                        |                              |  |
| Category <sup>6</sup>   | Citation of Document, <sup>10</sup> with indication, where appropriate, of the relevant passages <sup>17</sup> | Relevant to Claim No. <sup>18</sup>                                       |                         |                        |                              |  |
| P,Y   | US, A, 4,959,305 (Woodrum)<br>25 September 1990, see entire document.  | 2,5,8-12,14,15,<br>18,21-23,26<br>28-30,33,35,37<br>39,46,50-52,<br>54,57 |                         |                        |                              |  |
| Y   | US, A, 4,830,959 (McNeil et al.)<br>16 May 1989, see entire document.  | 1-57  |                         |                        |                              |  |
| Y   | US, A, 4,758,323 (Davis et al.)<br>19 July 1988, see entire document.  | 1-57  |                         |                        |                              |  |
| Y   | US, A, 4,224,125 (Nakamura et al.)<br>23 September 1980, see entire document.                                  | 1-57  |                         |                        |                              |  |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div> |  |   |                         |                        |                              |  |
| <b>IV. CERTIFICATION</b>  |  |   |                         |                        |                              |  |
| Date of the Actual Completion of the International Search <sup>2</sup>  |  | Date of Mailing of this International Search Report <sup>2</sup>          |                         |                        |                              |  |
| 24 March 1991   |  | 18 APR 1991   |                         |                        |                              |  |
| International Searching Authority: <sup>1</sup>   |  | Signature of Authorized Officer <sup>10</sup>                             |                         |                        |                              |  |
| ISA/US  |  | <br>Janelle D. Waack  |                         |                        |                              |  |

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

- I. Process and apparatus for performing process of detection of an analyte by a biosensor, classified in class 435, subclass 288 (claims 1-15, 24-33 and 36-57;
- II. A reagent classified in class 435, subclass 14 (claims 16-23, 34 and 35).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**